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Prions: Beyond a Single Protein

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SUMMARY

Since the term protein was first coined in 1838 and protein was discovered to be the essential component of fibrin and albumin, all cellular proteins were presumed to play beneficial roles in plants and mammals. However, in 1967, Griffith proposed that proteins could be infectious pathogens and postulated their involvement in scrapie, a universally fatal transmissible spongiform encephalopathy in goats and sheep. Nevertheless, this novel hypothesis had not been evidenced until 1982, when Prusiner and coworkers purified infectious particles from scrapie-infected hamster brains and demonstrated that they consisted of a specific protein that he called a "prion." Unprecedentedly, the infectious prion pathogen is actually derived from its endogenous cellular form in the central nervous system. Unlike other infectious agents, such as bacteria, viruses, and fungi, prions do not contain genetic materials such as DNA or RNA. The unique traits and genetic information of prions are believed to be encoded within the conformational structure and posttranslational modifications of the proteins. Remarkably, prion-like behavior has been recently observed in other cellular proteins—not only in pathogenic roles but

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also serving physiological functions. The significance of these fascinating developments in prion biology is far beyond the scope of a single cellular protein and its related disease.

INTRODUCTION

Prions, a term derived from the phrase "proteinaceous infectious particle" (1), are the pathogens that cause a group of fatal zoonotic transmissible spongiform encephalopathies (TSEs) also known as prion diseases. Although they belong to the class of neurodegenerative disorders that includes Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD), prion diseases affect both animals and humans. On the basis of early reports, perhaps the earliest known prion disease is scrapie, which was found on European farms during the 18th century (2). The English name scrapie is most likely derived from the word "scrape," which comes from the notion that afflicted sheep or goats are often found scraping off their coats since they suffer from pruritus. Interestingly, according to his analysis of the Chinese character for itchy or pruritis, 痒 or 瘙痒, Reed Wickner proposed that scrapie may have origins dating as far back as ancient China (2). As he indicated, the Chinese character for itchy (痒) is a combination of the key parts of the Chinese characters for disease (病) and sheep (羊)—indicative of a symptom seen in diseased sheep (病羊 in Chinese). Another interesting association is that the pronunciation of the Chinese character for itchy (痒) is the exact same as that of the Chinese character for sheep (羊). Since most Chinese characters have their own meanings and were developed 2,000 to 3,000 years ago, scrapie is believed to have existed during the time its character was being created (2). Although the infectious nature of scrapie was already surmised in the 18th century, its transmissibility was not proven experimentally until 1936 (3). Now scrapie is found almost worldwide and exists in two distinct forms, classical and atypical (4); each subtype exhibits distinct clinical, epidemiological, molecular, and histopathological features.

Apart from scrapie, there are several other animal prion diseases (Table 1), the most notable of which are chronic wasting disease (CWD) and mad cow disease. In 1984, mad cow disease, also known as bovine spongiform encephalopathy (BSE), first appeared in cattle in the United Kingdom (5); thereafter, it rapidly evolved into a major epidemic, and by 2004, more than 180,000 cases of BSE had been reported. Mad cow disease has been identified in 23 countries, including Canada and the United States. In addition to the initially identified and most common form of BSE, classical BSE (C-type BSE), two atypical types, named the H and L types, were also identified in 2004 (6–8). The discovery of the link between a new variant Creutzfeldt-Jakob disease (vCJD) in humans and the outbreak of mad cow disease brought prion diseases to the public eye in the mid-1990s (9). To date, approximately 200 cases of vCJD have been recorded (10).

CWD is a nonhuman prion disease that affects Rocky Mountain elk, moose, and certain species of North American deer, including white-tailed deer and mule deer. The disease is found in both free and captive populations and has been observed in at least 22 states in the United States and three Canadian provinces (11). Given that thousands of elk and deer are hunted and consumed each year, the possibility cannot be excluded that a cohort of people in North America may harbor CWD prions through the unintentional consumption of infected deer or elk meat. Nevertheless, there is no evidence that CWD has been transmitted to humans. Transmission studies have been unsuccessful in their efforts to cause disease in transgenic (Tg) mice expressing human PrP following intracerebral CWD inoculation (12–14).

Compared to other common neurodegenerative diseases such as AD and PD, prion diseases possess several unique characteristics. Not only are they transmissible to both animals and humans, but they are also highly heterogeneous. For example, the clinical and pathological characteristics of AD described in the first case by German physician Alois Alzheimer in 1907 (15) are still considered to be the diagnostic hallmarks of the disease. In contrast, the complexity of human prion diseases has been striking since its first description almost a century ago. In 1921, Alfons Maria Jakob erroneously considered a new malady (now known as Creutzfeldt-Jakob disease [CJD]) to be the same disease that was previously described by Hans Gerhard Creutzfeldt in 1920 (16–18). Although they represented different diseases, the names of Creutzfeldt and Jakob have been linked together ever since. To complicate matters further, only two of Jakob's five original cases actually satisfy the present diagnostic criteria for prion disease (18, 19). Nevertheless, it was more than half a century after the first report of CJD that the disease became grouped into the same category as scrapie (20).

In the 100 or so years since the original finding of CJD, other forms and subtypes of human prion diseases have joined this group, which now encompasses sporadic, inherited, and acquired forms of the disease (Table 1). On the basis of clinical histories and neuropathological features coupled with molecular typing, sporadic CJD (sCJD), the most prevalent human prion disease, can be further categorized into six molecular or five clinical subtypes (21). In addition to sCJD, there also exists a familial form of the disease that accounts for approximately 10 to 15% of all human prion diseases and is linked to >40 different mutations of the *PRNP* gene. Acquired forms of CJD include iatrogenic CJD, caused by accidental exposure to prions during medical or surgical procedures (22, 23), and vCJD, linked to the consumption of BSE-contaminated food (9). These account for approximately <2 to 5% of all prion diseases. Other main prion phenotypes include Gerstmann-Sträussler-Scheinker (GSS) syndrome, fatal insomnia (FI), and variably protease-sensitive prionopathy (VPSPr) (24–27) (Table 1). Also contained in this group is a disease known as kuru, a human prion disease that is restricted to the Eastern Highlands Province of Papua New Guinea and is acquired by participation in cannibalistic feasts (28, 29).

Despite their highly heterogeneous features, prion diseases are universally characterized by central nervous system (CNS) deposition of the aberrant scrapie form of prion protein (prions or PrP^{Sc}) (1, 30–32). The finding of PrP^{Sc} as a molecular hallmark of all TSEs has made it possible to definitively diagnose prion diseases at both the pathological and molecular levels. This finding has greatly facilitated the identification of the aforementioned novel animal and human prion diseases. Moreover, the discovery and further characterization of PrP^{Sc} have provided enormous insights into the nature of prions, the pathogenesis of prion diseases, and above all, the transmissibility of the diseases.

In contrast to viruses or bacteria, unprecedentedly, prions consist of amino acids but lack nucleic acids. Prion strains that give rise to varied phenotypes of prion diseases are believed to be associated with highly variable conformations of PrP^{Sc} (33, 34). Surprisingly, PrP^{Sc} is derived from the cellular prion protein (PrP^{C}) by a conformational misfolding event characterized by the transition of α-helixes into β-sheet structures (32). PrP^{C} is a membrane glycoprotein that is expressed mainly in the CNS but can also be

TABLE 1 Animal and human prion diseases and related pathogenic proteins[a]

Disease type or prion protein(s)	Disease(s)
Diseases	
Animal	Scrapie (sporadic or acquired), CWD (sporadic or acquired), BSE (sporadic or acquired), transmissible mink encephalopathy (sporadic or acquired), feline spongiform encephalopathy (sporadic or acquired), exotic ungulate encephalopathy (sporadic or acquired)
Human	Kuru (sporadic and acquired), CJD (sporadic, inherited, or acquired), GSS syndrome (inherited or sporadic), FFI (inherited or sporadic), VPSPr (sporadic or familial history)[b]
Prion protein(s)	
Aβ and Tau	AD (sporadic or inherited)
α-Synuclein	PD (sporadic or inherited), dementia with Lewy bodies (sporadic), multiple-system atrophy (sporadic)
Polyglutamine-containing proteins	HD (inherited); dentatorubro-pallidoluysian atrophy (inherited); spinal and bulbar muscular atrophy (inherited); spinocerebellar ataxia types 1, 2, 3, 6, 7, and 17 (inherited)
Tau, TDP-43	Frontotemporal dementia (sporadic or inherited), corticobasal degeneration (sporadic), progressive supranuclear palsy (sporadic)
SOD1, TDP-43	ALS (sporadic or inherited)

[a] Data cited from reference 349.
[b] No mutation found in PRNP but ~20% cases reported with family history.

found in other non-CNS tissues and organs (35). Its physiological functions remain ill defined; however, several studies have revealed that PrP^C may play a role in oxidative stress reduction, signal transduction, apoptosis regulation, cellular uptake or binding of copper ions, adhesion of the extracellular matrix, and the formation and maintenance of synapses (reviewed in reference 36).

Although the prevalence of BSE and BSE-related vCJD has receded, a future outbreak of prion disease due to interspecies or intraspecies transmission is still conceivable. Given the high incidence and prevalence of CWD in North America, the question of the possibility of transmitting CWD to humans remains. In addition to these epidemiologic concerns, there are many questions regarding prion formation and disease pathogenesis that remain unanswered.

CELLULAR PRION PROTEIN AND ITS INSOLUBLE FORM

PrP^C

PrP^C, from which the infectious prion agent is derived, is encoded by the prion protein gene *PRNP* located on 20p13 (37). This highly conserved gene is composed of three exons. The terminal exon, containing the open reading frame (ORF), encodes a 253-amino-acid sequence named PrP^C (where the superscript C means cellular) or simply PrP. Of the 253 amino acids, residues 1 through 22 constitute the N-terminal signal peptide, while residues 232 to 253 make up the C-terminal hydrophobic peptide. In the rough endoplasmic reticulum (ER), the PrP^C molecule is subjected to the following posttranslational modifications: removal of the N- and C-terminal signal peptides, addition of N-linked glycans at residues N-181 and N-197, formation of a disulfide bridge between residues C-179 and C-214, and addition of the glycophosphatidylinositol (GPI) anchor at residue 231 following cleavage of the C-terminal hydrophobic peptide (38–41) (Fig. 1). After it is synthesized and modified in the ER, mature PrP^C, consisting of 209 residues, is transported to the cell membrane via the Golgi body (Fig. 2). Residues 23 to 124 form the flexible N-terminal domain, and residues 125 to 228 make up a globular domain (Fig. 1). When properly assembled, the tertiary structure is composed of three α-helices and two antiparallel β-sheets in the C-terminal domain (42, 43) (Fig. 1). The N terminus contains five or six repeats of eight glycine-rich residues (PHGGGWGQ) forming the octapeptide repeat region. These octapeptide repeats bind Cu(II) (Fig. 1) and several other divalent cations (44–46), thereby providing a mechanism for PrP's role in reducing oxidative damage (47).

On a molecular level, PrP^C is located mainly on cellular membranes, although different topological forms, including transmembrane forms, have been identified. PrP^C is most notably found in the CNS but is also expressed in several tissues throughout the body, including the heart, muscle, lymphoid tissues, kid-

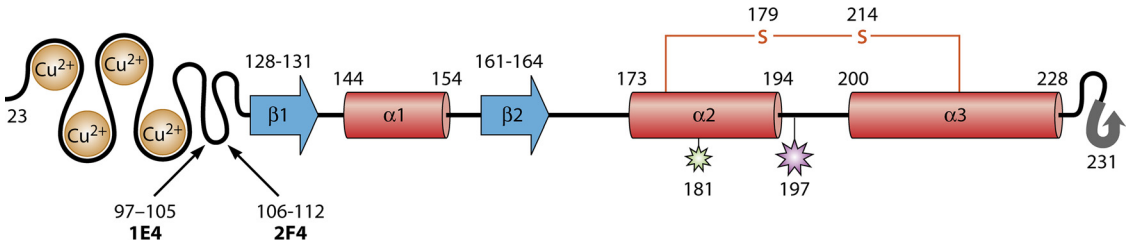

FIG 1 Schematic diagram of the nuclear magnetic resonance-derived structure, posttranslational modifications of human PrP^C, and epitopes of anti-PrP antibodies. Mature human PrP contains 209 amino acids. It consists of a flexible N-terminal domain containing four copper-binding octapeptide repeats and a folded C-terminal domain containing two β-sheets and three α-helical structures. The cysteines at positions 179 and 214 form a disulfide bond between the α2 and α3 domains. Two N-linked glycosylation sites are at residues 181 and 197, and the GPI anchor is linked to residue 231. The epitopes of anti-PrP antibodies 1E4 and 3F4 are located at residues 97 to 105 and 106 to 112, respectively.

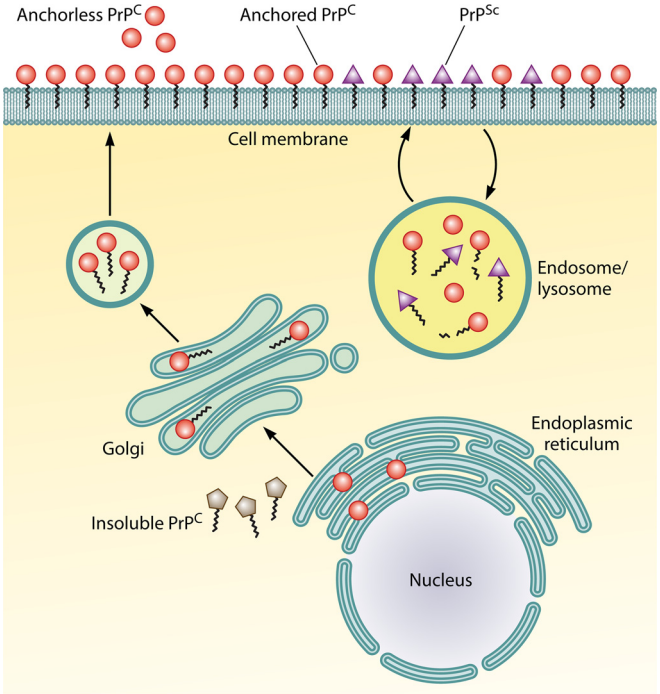

FIG 2 Biosynthesis, trafficking, cleavage, and conversion of cellular PrP. PrP^C is synthesized and posttranslationally modified in the ER. It is then transported to the cell membrane after further modifications in the Golgi body. In the ER, the protein undergoes cleavage of the N- and C-terminal signal peptides, followed by the addition of N-linked glycan moieties at two sites, as well as the GPI anchor. Lastly, a single disulfide bond is formed. After reaching the cell membrane, some PrP^C is internalized into endosomes, while most of the PrP molecules are recycled to the cell membrane. A limited amount of the endocytosed PrP is subjected to cleavage at residue 110. The membrane-anchored PrP may be released into the extracellular space by cleavage within the GPI anchor. Conversion of PrP^C into PrP^{Sc} has been reported to occur in both cell membranes and endosomes or lysosomes. It is most likely that $iPrP^C$ accumulates around the nucleus.

neys, gastrointestinal tract, skin, and endothelium (35, 48, 49). Among the CNS cell types, PrP^C has been identified in neurons, extraneural tissues, and glial cells (35, 50–52). In addition, PrP^C is found with the same frequency on pre- and postsynaptic neurons. Moreover, within the CNS, PrP^C expression occurs in particular regions of the brain, including the olfactory bulb, striatum, hippocampus, and prefrontal cortex (53, 54). Quantitative ultrastructural studies employing electron microscopy in the mouse CA1 and hippocampal dentate gyrus have revealed prion proteins to be localized to the secretory pathway, with a preference for late endosomal compartments and the plasma membrane (55, 56). Furthermore, PrP^C is found in the cytosol of neocortical, hippocampal, and thalamic neurons but is absent from the cytosol of cerebellar neurons.

Interestingly, studies expressing PrP^C in the cytosol have shown that such expression is neurotoxic and may be mechanistically implicated in prion diseases (57). Within the CNS, PrP^C was observed to colocalize with dopaminergic neurons, suggesting its involvement in dopamine homeostasis and, possibly, its role in the neuropathogenesis of prion disease (58).

The physiological functions of PrP^C are largely undetermined. A limited understanding of PrP^C and its functions comes from studies using animal models, *in vitro* experiments, and epidemiological research. PrP^C binds to Cu(II) with high affinity, suggesting a role as a copper transporter (44). Because of its ability to chelate Cu(II), PrP^C has been implicated as an antioxidant that is potentially capable of reducing cellular reactive oxygen species (47). Ford and colleagues (52) showed that PrP^C expression is particularly increased in GABAergic neurons; however, it is also present at some level in noradrenergic, glutamatergic, cholinergic, and serotonergic neurons, suggesting its involvement in neurotransmission.

PrP^C may also be involved in cell-cell adhesion, modulation of cell-cell junctions, and signaling *in vivo* (49). PrP^C has been shown to interact with laminin, a structural basement membrane glycoprotein, thereby contributing to the regulation of neuritogenesis (59). Additionally, PrP^C was found to be a component of desmosomes in the intestinal epithelium (reviewed in reference 49). Furthermore, it may serve as a positive regulator in cell differentiation and in neuronal precursor proliferation throughout neurogenesis (60).

Studies involving PrP^C knockout mice have failed to show any consistent deficits. These Tg mice are clinically normal, with no obvious abnormal developmental phenotypes. However, some studies have shown several neurologic aberrancies such as circadian rhythm dysregulation, deficits in cognition and olfaction, and immunologic alterations (reviewed in reference 49). Moreover, increased levels of NF-κB, decreased Cu/Zn superoxide dismutase activity, and reduced p53 activity have been reported (61). The latter finding suggests a role for PrP^C in cancer biology. Consistent with this idea, PrP^C is increased in gastric carcinoma and confers multidrug resistance via P-glycoprotein upregulation (62).

Insoluble PrP^C Aggregates

PrP^C has been historically characterized as soluble, monomeric, and protease sensitive (32). It is abundant in the CNS of uninfected humans and animals. Within the last decade, however, an insoluble conformation of PrP^C has been identified in the brains of normal, healthy humans, as well as cultured neuronal cells (63, 64). Found in monomeric, dimeric, oligomeric, and multimeric forms, such species may constitute up to 25% of the total PrP^C expressed in the CNS (63). This novel isoform, termed insoluble PrP^C ($iPrP^C$), is identified by Fd gene 5 protein (g5p) and sodium phosphotungstate—agents that bind only to misfolded PrP^{Sc} and not PrP^C (65, 66). Its avidity for g5p, a single-stranded DNA binding protein, suggests a possible association with nucleic acids, a consideration previously given to PrP^{Sc} (67–69). Interestingly, in the same specimens found to have $iPrP^C$, proteinase K (PK)-resistant PrP fragments were isolated and were recognized by g5p. Furthermore, a fraction of $iPrP^C$ was resistant to PK degradation, even at high concentrations (63). This PK-resistant $iPrP^C$ has a high affinity for anti-PrP monoclonal antibody 1E4 but a low affinity for the widely used 3F4 antibody.

Our previous study demonstrated that cell-expressed human wild-type PrP (PrP^{Wt}) or mutant PrP (PrP^{Mut}; including the naturally occurring mutation PrP^{T183A}, PrP^{F198S}, or PrP^{V180I}) contains $iPrP^C$ that is detectable with the 1E4 antibody; however, the 3F4-detected, PK-resistant PrP fragment is observed only in cells expressing PrP^{Mut} (64, 70). This study favors the hypothesis that the pathological conversion of PrP^C occurs more efficiently in PrP^{Mut} than in PrP^{Wt}, and protein aggregation is the major molecular event accompanying the conversion of PrP^C into PrP^{Sc}.

Using immunofluorescence microscopy, we observed that 1E4-detected PrP is localized mainly in the cytoplasm around the nucleus and is PK resistant (70, 71) (Fig. 2). Taken together, these studies suggest that the PrP species detected preferentially with 1E4 may be different from those detected with 3F4, although the two antibodies have epitopes adjacent to each other on PrP (PrP residues 97 to 105 for 1E4 and PrP residues 106 to 112 for 3F4) (63, 72) (Fig. 1). Moreover, it is possible that these species have a unique localization and conformation. Interestingly, using a combination of approaches, including ultracentrifugation and gel filtration (fast protein liquid chromatography), we consistently isolated not only $iPrP^{C}$ aggregates but also soluble PrP^{C} oligomers from the healthy human brain (66). Therefore, similar to PrP^{Sc}, PrP^{C} may also possess chameleon-like conformations (71).

The molecular function of $iPrP^{C}$ and its involvement in prion pathogenesis remain unknown. Remarkably, 1E4-detected $iPrP^{C}$ seems to be associated with a new prion disease in humans. Employing the 1E4 antibody, we recognized a novel PrP^{Sc} strain in an unusual sporadic human prion disease termed VPSPr (27, 73). Although VPSPr exhibited two PK-resistant PrP^{Sc} (PrP^{res}) fragments detected by 3F4 in patients with Met/Met or Met/Val polymorphism at codon 129 of PrP, the typical five-stair ladder-like profile of PrP^{res} was detectable with 1E4 (27, 74). Moreover, using this antibody, we recently found that the same peculiar ladder-like PrP^{res} is also detected in familial CJD linked to the PrP^{V180I} mutation (70). The C- and N-terminal cleavage sites of this peculiar PK-resistant PrP fragment from VPSPr was determined by antibody mapping, which showed that the N-terminal PK cleavage sites were contained within residues 99 to 101, while the C-terminal cleavage sites were between residues 152 and 157 (75). It should be noted that PrP^{Sc} detected in VPSPr and $fCJD^{V180I}$ has the same 1E4 immunoreactivity as $iPrP^{C}$ found in uninfected brains.

Unexpectedly, not only has $iPrP^{C}$ been implicated in prion disease, but it may also be involved in AD pathogenesis. By using coprecipitation assays with AD brains and peptide membrane arrays, we observed that $iPrP^{C}$ is the main PrP conformer that may interact with amyloid β42 (Aβ42) aggregates (76)—a finding that was later confirmed by Dohler and coworkers (77).

Beyond neurodegenerative diseases, PrP^{C} aggregates may play a role in other seemingly unrelated diseases. Interestingly, cytosolic PrP^{C} aggregates were found in β-islet pancreatic cells in diabetes-prone rats (78). In rodent models of hypoxic-ischemic injury, somal PrP^{C} aggregates were observed in the penumbra of hypoxic damage (79); moreover, knockout mice devoid of PrP^{C} were more susceptible to strokes, as evidenced by larger infarct sizes than those of wild-type mice (79, 80). In an earlier study, overexpression of PrP^{C} via replication-defective adenoviral vectors into an ischemic rat brain significantly reduced cerebral infarction volumes (81). Taken together, these studies imply that PrP^{C} generates a neuroprotective response in the presence of cerebral ischemia. While the precise molecular mechanisms are unclear, in knockout mice lacking PrP^{C}, the antiapoptotic phosphatidylinositol 3-kinase/Akt pathway has been shown to be downregulated, while caspase-3 activation has been shown to be upregulated following ischemic injury. The combination of such dysregulation resulted in exacerbated cellular injury *in vivo* (80). Other studies employing $Prnp^{-/-}$ mice have revealed an upregulation in several cytosolic signaling pathways, including ERK-1/-2, STAT-1, and caspase-3, after induction of cerebral ischemia (82).

Given the fact that soluble PrP^{C} is undetectable on tissue sections treated with both formic acid and microwave heating, while misfolded PrP^{Sc} is still detected on these sections after these treatments, we hypothesized that the PrP species detected in the stroke brain are characteristic of $iPrP^{C}$ (given resistance to tissue ischemia). We further compared PrP staining by immunohistochemistry (IHC) in brain sections obtained after an ischemic stroke. As expected, PrP staining was not observed in the healthy controls (Fig. 3A), whereas intense PrP staining was evident in the sCJD brain sections (positive controls) (Fig. 3D). In contrast, weak-to-moderate PrP immunostaining was also observed in the infarct areas of brains after an ischemic stroke (Fig. 3B and C). The fact that PrP species detected in the ischemic stroke brain are resistant to treatment with formic acid and heat may be attributable to the conformational transition of the molecule rather than a rise in protein expression. It is certainly possible that PrP species found in the infarct regions have a high tendency to form aggregates and become protease resistant because of a hypoxia/ischemia-induced low-pH environment. Indeed, we have previously demonstrated *in vitro* that acidic pH induced the conversion of soluble PrP^{C} into $iPrP^{C}$ in human brain homogenates (83).

PRIONS AND DIVERSE STRAINS

Prions

It is generally accepted that the critical molecular phenomenon in prion disease pathogenesis is the conformational transition from α-helix-rich PrP^{C} to a structure rich in β-sheets, PrP^{Sc}. This β-sheet-rich structure has properties that allow for greater stability and are responsible for the ability of PrP^{Sc} to form aggregates; such PrP^{Sc} aggregates are capable of forming amyloid fibrils. Part of the fibrils can break off and act as a template for the recruitment of additional PrP^{C} molecules. Only PrP^{C} molecules with a sequence identical to that of infectious PrP^{Sc} molecules are incorporated into the growing amyloid fibril. These aggregates are located extracellularly and in other vesicular compartments within the cell (84).

Studies employing infrared spectroscopy and circular dichroism spectroscopy determined that PrP^{C} is composed largely of α-helices (42%) with a fractional content of β-sheets (3%) (85). In contrast, PrP^{Sc} is composed largely of β-sheets (>43%) with N-terminally truncated PrP^{Sc} containing an increased fraction of β-sheet content (>54%) (85, 86). PrP^{Sc} is structurally polydisperse and hence is difficult to crystallize. Although numerous models have been suggested, the definitive structure of PrP^{Sc} has not yet been elucidated, given its proclivity to aggregate; moreover, current models have been discordant with experimental data (87).

The general transformation of PrP^{C} to its amyloidogenic form, PrP^{Sc}, is poorly understood and is a highly debated topic. Given the experimental failure to recapitulate this conversion with protein-only substrates, a large body of evidence suggests that other macromolecules may be involved (88, 89). The precise components necessary and sufficient to mediate this process *in vivo* remain to be elucidated, and not surprisingly, an increasing number of cofactors have been identified as potentially contributing to the conversion event. Given the association of PrP^{C} with lipid rafts, several studies have implicated lipid rafts themselves as directly involved in the conversion of PrP^{C} to PrP^{Sc} (90). Additionally, heparan sulfate proteoglycans (HSPGs) and their glycosamino-

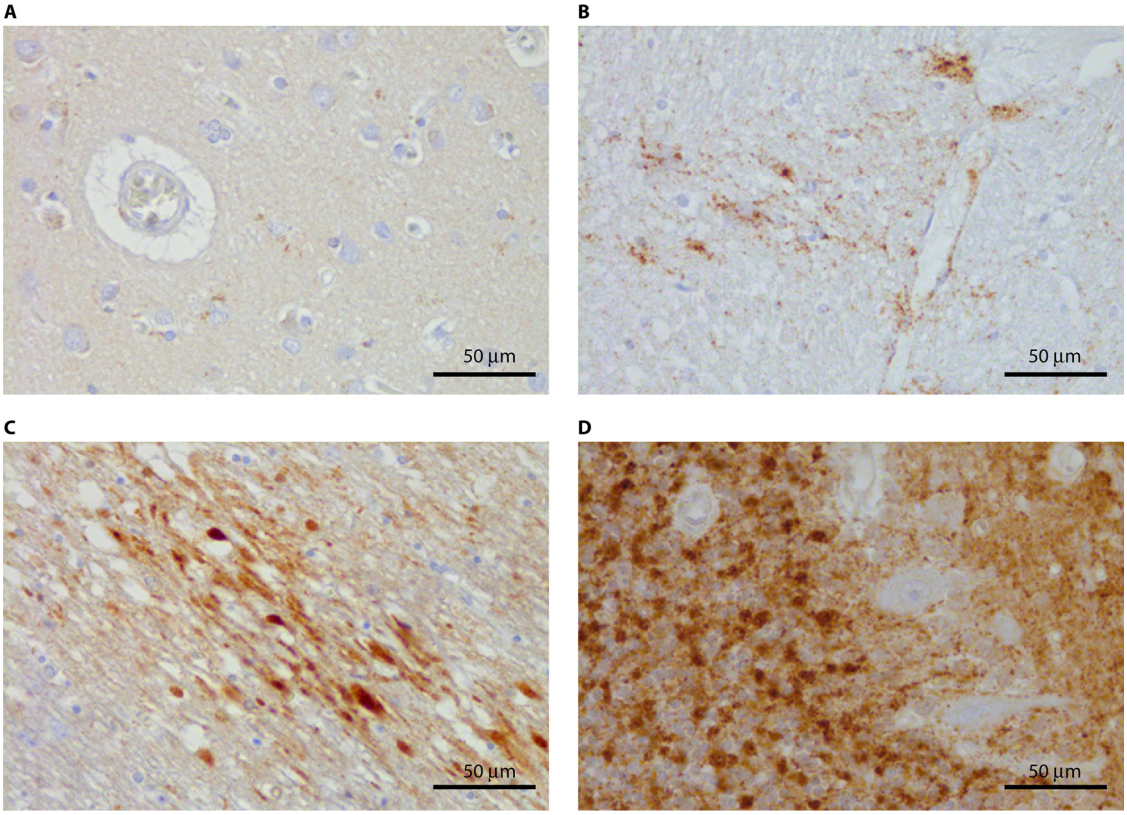

FIG 3 IPrP^C detected in ischemic-stroke brain tissue by IHC. (A) Healthy human brain sample. (B, C) Brain samples from ischemic-stroke patients. (D) Brain sample from a patient with sCJD. IHC was done with anti-PrP antibody 3F4.

glycan side chains may be involved in PrP^C metabolism and conversion (91). Specifically, it has been proposed that the neuronal GPI-anchored HSPG glypican-1 may bring PrP^C and PrP^{Sc} in close proximity to lipid rafts to facilitate the pathogenic conversion (92). Moreover, a negative regulator of Wnt signaling, Notum, may facilitate the cleavage of glypicans 1, 3, 5, and 6, suggesting a modulatory role in PrP^C conversion. Other macromolecules like GM1 ganglioside may also be involved, as it was shown to be a ligand for the C terminus of PrP^C (93). By virtue of their interaction with PrP, several other molecules may facilitate the conversion of wild-type PrP^C, including laminin receptor precursor 1, neural cell adhesion molecules, anionic lipids, and copper ions (94).

Molecules that have been found to colocalize with scrapie-associated fibrils include α-linked polyglucose (similar to glycogen), nucleic acids such as poly(A) RNA, ferritin, CaMKII, actin, and tubulin (94). In yeast, heat shock proteins have been established as a potential cofactor (95). In a landmark study by Wang and colleagues, infectious PrP^{Sc} particles were generated by using anionic phospholipid 1-palmitoyl-2-oleoylphosphatidylglycerol and RNA (96). Furthermore, the single cofactor endogenous phosphatidylethanolamine (PE) in the absence of nucleic acids was sufficient to facilitate the conversion of PrP^C to infectious PrP^{Sc} (97).

While different cofactors have been required for the *in vitro* generation of infectious prions as mentioned above, in the absence of cofactors, recombinant PrP (rPrP) aggregates cause prion disease after inoculation into Tg mice or wild-type hamsters (98–100). This key finding strongly supports the "protein-only" hypothesis.

Prion Strains

Prions are capable of transmitting a variety of prion diseases with variegated phenotypes. An interesting concept known as "prion strains" arises when such phenotypes are discussed. Prion strains are defined as infectious particles that possess distinctive histopathological and clinical features when inoculated into syngeneic hosts. Various traits among strains include regional deposition of the prion protein, unique incubation times, characteristic neurotropism, and particular distribution of CNS lesions (101). It is hypothesized that PrP^{Sc} adopts several conformations, with each conformation (or strain) capable of precipitating a particular disease; moreover, each specific strain is thought to exhibit distinctive biochemical properties (34, 102). Furthermore, the interaction of such strains with host polymorphisms in codon 129 of the *PRNP* provides for a plethora of phenotypes. It is not surprising that such "strains" may be found in other neurodegenerative conditions such as AD (103–105).

The earliest evidence of prion strains comes from preliminary studies on scrapie-infected mice (106, 107). At that time, five such scrapie species were identified on the basis of various degrees of spongiform degeneration and the distribution of brain lesions. As the prion protein's molecular identity came to be known, another defining feature of prion strains arose. It became evident that glycosylation states and the relative proportions of unglycosylated, monoglycosylated, and diglycosylated forms contribute to prion diversity (108, 109). In the PrP^C transcript, asparagine glycosylation can exist at residues 181 and 197. From Western blot analysis of PrP^C glycoforms, it was proposed that unique proportions of

glycosylated forms could give rise to different PrP^{Sc} strains (101, 110). Using glycoform-specific ratios, such differences were used to distinguish sCJD and sFI phenotypes with the same underlying polymorphism (111).

Another characteristic used to classify strains is the electrophoretic mobility of the PK resistance fraction (PrP^{res}). In 1992, Richard Bessen and Richard Marsh first identified two different PK-resistant PrP^{Sc} fragments (two prion strains) in Syrian hamsters with transmissible mink encephalopathy (112). These two strains were classified as the Hyper and Drowsy. Migration of the PK-resistant PrP^{Sc} fragment varied between the two strains: Hyper (hyperexcitability and cerebellar ataxia) slowly migrated at ~21 kDa on the gel, while Drowsy (progressive lethargy) quickly migrated at ~19 kDa. Presumably, different conformations of PrP^{Sc} have different PK cleavage sites exposed; upon PK digestion, unique PrP^{res} fragments are generated, thereby explaining the various degrees of mobility. Interestingly enough, different PK-resistant PrP^{Sc} fragments similar to those of Hyper and Drowsy were detected in various human prion diseases, including CJD, fatal familial insomnia (FFI), and kuru (113–115). The molecular basis of these size differences among PK-resistant PrP^{Sc} core fragments was later investigated by N-terminal sequencing (116). Two primary N-terminal cleavage sites were identified: residue G82 for PrP^{Sc} type 1, which has an unglycosylated PrP migrating at 21 kDa on gels, and residue S97 for PrP^{Sc} type 2 migrating at 19 kDa.

Further evidence for prion typing comes from a study on FFI and sCJD in which transmission of infected human brain homogenates (either FFI or sCJD) to Tg mice expressing chimeric human-mouse PrP resulted in the recovery of specific human fragments in mice, 21 kDa in sCJD and 19 kDa in FFI (117). More recently, using knock-in Tg mice expressing human PrP carrying one of three different 129 polymorphisms (129MM, 129MV, or 129VV), Bishop et al. identified four of the six prion strains that were reported by Parchi and coworkers in sCJD (115, 118). In the bioassay performed by Bishop et al., of the six types of sCJD, PrP^{Sc}MM1 was indistinguishable from PrP^{Sc}MV1, while PrP^{Sc}MV2 could not be distinguished from PrP^{Sc}MM2 (118).

The above molecular typing of PrP^{Sc} and classification of sCJD provide insights into the molecular underpinnings that contribute to the heterogeneity of prion diseases. However, this molecular typing of PrP^{Sc} is mainly dependent on the detection of PK-treated PrP^{Sc} core fragments by nondiscriminatory anti-PrP antibodies such as 3F4. Given that the prion strain hypothesis is based on the variable conformations of PrP^{Sc}, PK identification and single-antibody-dependent molecular typing were considered to be inefficient methods of studying PrP^{Sc} conformations (119). Indeed, the N-terminal sequencing data revealed that PK digestion generates a mixture of PrP^{Sc} fragments with ragged N termini even from a solitary brain (116). This suggests the presence of multiple conformations of PrP^{Sc} in a single infected brain. In light of this, it is possible that there are other factors that mediate protein conformation that may not be fully appreciated. Collinge and coworkers identified three (instead of two) types of PrP^{Sc} in sCJD based on gel mobility (120, 121). The additional PK-resistant PrP^{Sc} fragment (120) migrated slower than PrP^{Sc} type 1 identified by Parchi and colleagues (115).

As evidenced above, co-occurrence of PrP^{Sc} subtypes has been reported in the same brain in approximately 30% of the cases tested (122). More specifically, with type 1-specific antibodies, PrP^{Sc} type 1 was detected in vCJD and in all sCJD patients classified as CJD type 2 (123). With a conformation-dependent immunoassay, more PrP^{Sc} strains were identified in experimentally infected animals (102), although some of them may have had the same gel mobility. Finally, Uro-Coste et al. identified four distinct PrP^{Sc} molecular subtypes in iCJD and sCJD patients, regardless of the underlying 129 polymorphism and the PrP^{Sc} isoform (124).

Prion strains have also been described in nonhuman TSEs, including CWD, BSE, and scrapie. Two prominent strains of CWD have been described in Tg mice expressing cervid PrP^{Sc}, CWD1 and CDW2, although they have identical electrophoretic and glycoform profiles/ratios (125). CWD1 is characterized by symmetric hippocampal deposits and short incubation times, while CWD2 is characterized by asymmetric cervid PrP^{Sc} deposition and long incubation times. Among deer and elk, differences in the primary structures at codon 226 account for the existence of prion strains (125). Further CWD strains have been identified in ferrets (126). As with human prionopathies, polymorphisms at *PRNP* also modulate disease susceptibility and CWD incubation periods (127).

By using the PrP^{res} electrophoretic profile as a strain marker, three prominent BSE strains have been identified: classic BSE (C type), L type, also known as bovine amyloidotic spongiform encephalopathy, and higher type (H type), named appropriately for its higher molecular mass than the C type (6). The H-type strain has additional cleavage sites, resulting in two PrP^{res} fragments of various sizes upon PK treatment (128). Additionally, H-type BSE contains more unglycosylated PrP^{res} and lower levels of diglycosylated PrP^{res} (128).

Similar to BSE, scrapie in sheep has strains characterized by low (L type) and high (H type) molecular masses. The predominant form has a high molecular mass and is unglycosylated (129, 130). Moreover, among scrapie species, the glycosylation patterns and molecular size of the unglycosylated form are not unique (129). When a conformation-dependent immunoassay was used, two distinct strains were identified on the basis of differences in sensitivity to PK denaturation (131). Interestingly, more than a decade ago, several cases of scrapie with atypical PrP genotypes and distinct neuropathological features were identified in Norway (4). Termed Nor98, this distinct prion disease is manifested by ataxia and is characterized by two unglycosylated PrP^{res} fragments with low molecular masses (132). Remarkably, the physiochemical properties of Nor98 PrP^{Sc} have many characteristics similar to those of the GSS syndrome and VPSPr (75). In addition, underlying polymorphisms at residues 141 and 154 of PrP have been linked to a greater risk of Nor98 transmission (133).

Protease-Sensitive Prions

Infectious pathogenic PrP^{Sc} traditionally refers to the PrP molecule that is resistant to PK digestion ($rPrP^{Sc}$) (30). However, there exists a PK-sensitive PrP^{Sc} ($sPrP^{Sc}$) that was originally found by Safar and coworkers with a conformation-dependent assay (102). By employing a PrP^{Sc}-specific antibody-based Western blotting assay, we were able to show that PrP^{Sc} from sCJD is composed of both $sPrP^{Sc}$ and $rPrP^{Sc}$; furthermore, a large portion of the PrP^{Sc} in GSS syndrome is PK sensitive (65). Safar et al. claimed that $sPrP^{Sc}$ may make up a significant fraction (90%) of the entire PrP molecule (134). The role of $sPrP^{Sc}$ in the pathogenesis of prion disease remains incompletely understood. However, by conventional Western blot analysis, we have revealed that VPSPr-129VV is abundant in $sPrP^{Sc}$ but is virtually devoid of $rPrP^{Sc}$ types 1 and

2 (27, 73). Moreover, our recent study of familial CJD cases linked to 144-bp insertion mutations asserts that $sPrP^{Sc}$ is the primary constituent of cerebellar PrP patches and that $sPrP^{Sc}$ is adequate in potentiating prion toxicity (135).

Choi and his colleagues were able to show that $sPrP^{Sc}$ aggregates from sCJD patients are taken up and subsequently degraded by astrocytes (136). Furthermore, $rPrP^{Sc}$ is subjected to similar astrocytic processing, suggesting that astrocytes are not able to differentiate between PK-sensitive and PK-resistant fractions. The ability of $sPrP^{Sc}$ and $rPrP^{Sc}$ to behave similarly implies that $sPrP^{Sc}$ is neurotoxic, likely precipitating pathology in a manner related to that of resistant prion species. Moreover, these $sPrP^{Sc}$ species may play an important role in modulating disease pathogenesis. In fact, the concentration of $sPrP^{Sc}$ has been shown to be correlated with prion incubation periods and disease progression rates (102, 137, 138).

A thermostable neutral metalloproteinase enzyme, thermolysin, has been shown to isolate both sensitive and resistant PrP^{Sc} fractions in rodent and human brains (139, 140). However, Pastrana et al. devised a method to isolate only $sPrP^{Sc}$ by differential centrifugation (141). By employing these isolation methods, they were able to determine that $sPrP^{Sc}$ has an infectivity comparable to that of $rPrP^{Sc}$; moreover, the two species have similar incubation times. Not surprisingly, such infectivity is effectively lost upon treatment with PK. Furthermore, like $rPrP^{Sc}$, $sPrP^{Sc}$ is capable of being amplified through a seeding mechanism commonly referred to as protein misfolding cyclic amplification (PMCA). In addition, the monomers that make up both molecules have similar structural properties, as determined by matrix-assisted laser desorption ionization and Western blotting. Collectively, these findings suggest that their differences lie in the size of particles (fewer multimers allowing for increased PK sensitivity) and not in the biochemical structure of the aggregates (141, 142). Moreover, the aforementioned studies have further demonstrated that different prion strains possess unique ratios of sensitive to resistant PrP^{Sc}.

Toxicity and Infectivity of Prion Protein Aggregates

The causative neurotoxic agent in prion disease remains to be elucidated. It is unclear whether the monomeric or oligomeric subunits or the interaction of subunits and additional components are required for neurotoxicity. While PrP^{Sc} is widely regarded as the pathogenic agent, for several reasons, there are additional complexities that arise with this hypothesis (143). Earlier studies introducing PrP^{Sc} into the CNS of PrP knockout mice demonstrated that such animals do not undergo neurodegeneration (144, 145). Furthermore, there are several subclinical prion disease phenotypes that have been experimentally detected in animals with substantial levels of PrP^{Sc}; however, these animals remain asymptomatic throughout their lives (146, 147). On the other hand, mice exposed to BSE prions remained devoid of PrP^{res} in the CNS despite being clinically symptomatic (148).

Such findings have also been recapitulated in human prion diseases. FFI or GSS syndrome linked to the A117V mutation exhibits striking clinical manifestations of prion disease but reveals small or even undetectable quantities of $rPrP^{Sc}$ (25, 149). In addition, there exist numerous inherited prion diseases not recapitulated in laboratory animals from which no PrP^{Sc} has been isolated (150–152).

Several potentially toxic PrP isoforms, including transmembrane, cytosolic, and PK-sensitive forms, have been observed in humans and prion-infected Tg rodents. Studies by Ma and Lindquist (153) showed that PrP^{C} can accumulate in the cytoplasm when proteasomal activity is inhibited. Furthermore, PrP^{C} is capable of forming protein aggregates in conjunction with Hsc70. Once in the cytosol, PrP^{C} can also adopt a PrP^{Sc}-like conformation that has been shown to be directly neurotoxic *in vitro* and *in vivo* (57).

Studies employing PG14 mice, Tg mice exhibiting a mouse homologue of familial CJD in humans, show that such mice undergo progressive neurodegeneration characterized by an accumulation of mutant PrP. This PrP shows similarities to PrP^{Sc} and is detergent insoluble, yielding a PrP 27-30 fragment (154). Similar findings were obtained with Tg mice overexpressing the murine homologue of the P102L mutation associated with GSS syndrome (151) and in Tg mice overexpressing the murine homologue of the D178N/V129 mutation associated with familial CJD (155). Additional studies overexpressing wild-type PrP^{C} in Tg mice demonstrated that such mice develop a type of neurodegeneration clinically manifested as paresis and tremor. These Tg mice accrue punctate cerebellar PrP^{C} aggregates that are mildly protease resistant but are surprisingly noninfectious (156).

The most infectious PrP species have been observed to have molecular masses ranging from 300 to 600 kDa, while oligomers of five or fewer PrP molecules or large aggregates display limited infectivity (157). This observation indicates that prion infectivity may be associated with the oligomeric state of PrP^{Sc}.

ROLE OF GLYCANS IN PRIONS

Differences in Composition of Glycans between PrP^{C} and PrP^{Sc}

Glycosylation variability is believed to be a major contributor to the heterogeneity of prion proteins (158). For instance, >400 different forms of PrP^{Sc} can be derived from the diversity of oligosaccharide structures (159). On the prion protein, there exist two conserved N-glycosylation sites, Asn181IleThr and Asn197PheThr. PrP^{C} exists in four glycoforms, including a diglycosylated form, two monoglycosylated forms (one at each site), and an unglycosylated form. Studies of Syrian hamsters showed that PrP^{C} and PrP^{Sc} exhibit the same group of >50 biantennary, triantennary, and tetra-antennary N-linked oligosaccharides (160). It has been observed that approximate 48% of the sugars are neutral, 20% are monosialylated, 18% are disialylated, 10% are trisialylated, 3.5% are tetrasialylated, and 0.5% contain five sialic acids (160). However, the proportions of individual glycans vary between the two forms. PrP^{Sc} exhibits fewer glycans with bisecting GlcNAc residues and more triantennary and tetra-antennary oligosaccharides. More specifically, the proportion of bisected glycans is 11% smaller in PrP^{res} than in PrP^{C}, and the proportions of triantennary and tetra-antennary glycans are 5 and 10% greater, respectively (160). The cause of this variability between the glycosylation patterns of the two molecules remains unclear, but Rudd and colleagues have proposed that the changes may result from decreased activity of a Golgi glycosyltransferase (*N*-acetylglucosaminyltransferase III; GnTIII) in PrP^{Sc}-forming cells (160, 161). Such enzymatic modulation suggests that aberrant glycosylation contributes to the pathogenesis of prion disease.

On the basis of sensitive mass spectrometry, glycan differences between the two glycosylation sites of PrP^{Sc} from mouse brains infected with the ME7 scrapie strain have been reported (162).

Although the major glycoforms identified at Asn-180 (equivalent to hamster or human 181) are also present at Asn-196 (equivalent to hamster or human 197), there are many other minor neutral components at Asn-180 that are not detected at Asn-196. For example, Asn-196 contains more trisialylated, triantennary, and tetra-antennary glycans than Asn-180. The majority of the glycoforms at Asn-180 are biantennary and triantennary structures falling within a molecular mass range of approximately 1,660 to 2,340 Da, whereas the major glycan components at Asn-196 have molecular masses ranging from approximately 2,000 to 3,020 Da (162).

Effect of Glycans on Prion Formation

It is widely believed that glycosylation increases the stability of proteins (163), as an increased amount of oligosaccharide may provide for a higher free-energy barrier, thereby preventing the pathological conformational transition to PrP^{Sc} (161). This hypothesis is concordant with the observation that the inhibition of glycosylation initiates or accelerates prion protein formation. For instance, of the di-, mono-, and unglycosylated PrP^{Sc} isoforms, the intensity of the diglycosylated PrP^{res} band on the gel is often the smallest in classic sCJD, suggesting that most of the diglycosylated PrP is not converted to PrP^{Sc}. Furthermore, treatment with tunicamycin to inhibit N-glycosylation facilitated PrP^{Sc} formation in scrapie-infected cells (164) and promoted the acquisition of PrP^{Sc}-like properties in transfected cells (165). Deletion or alteration of glycosylation by naturally occurring mutations is frequently observed in familial prion diseases. For example, in fCJD PrP^{V180I} or PrP^{T183A}, the PrP^{res} fragment lacks the diglycosylated form (70, 166–168). In the latter case, the T183A polymorphism results in the loss of a glycosylation site, potentially explaining a mechanism for altered glycosylation; however, the mechanism for aberrant glycosylation in PrP^{V180I} is unclear. Moreover, other mutations, including D178N, E200K, and F198S, have been linked to distorted ratios of the three PrP glycoforms. However, despite these findings, it should be noted that the effect of altered glycosylation on PrP^{Sc} formation in familial prion diseases may be minimal, as prion formation in such diseases has often been attributed to underlying amino acid substitutions (161).

The glycan's involvement in the pathophysiology of prionopathies may be underestimated, given our novel finding of a glycoform-selective prion formation pathway in VPSPr (27, 70, 74). Although no mutation has been identified in the ORF of *PRNP*, VPSPr is characterized by the CNS deposition of PrP^{Sc} through a glycoform-selective prion formation pathway similar to that of $fCJD^{V180I}$, which lacks diglycosylated glycoforms and glycoforms monoglycosylated at Asn-181 (70, 74) (Fig. 4). Moreover, both diseases contain a PrP^{res} that exhibits a ladder-like electrophoretic banding pattern when detected with an antibody directed against the PrP area next to the epitope of the 3F4 antibody (Fig. 1). Like $iPrP^{C}$, PrP^{Sc} from VPSPr exhibits low affinity for 3F4 but high affinity for 1E4, suggesting a possible association between the two conformers. This finding of glycoform-selective prion formation is the first evidence that glycosylation is critically implicated in the development, assortment, and strain characterization of prions. Furthermore, it is certainly possible that VPSPr is actually the first prion disease that is caused by alterations in glycosylation.

Interestingly, mutations affecting glycosylation sites in PrP^{C} can lead to an accumulation of $iPrP^{C}$. With neuroblastoma cells expressing mutant human PrP^{T183A} and PrP^{F198S}, cells that expressed the T183A mutation were found to have an increase in the aggregation of mutated PrP, as well as $iPrP^{C}$. The PrP^{T183A} mutant form was devoid of glycosylation at residue 181 and exhibited altered glycosylation at residue 197. With regard to the PrP^{F198S} mutation, there was altered glycosylation at both glycosylation sites (169).

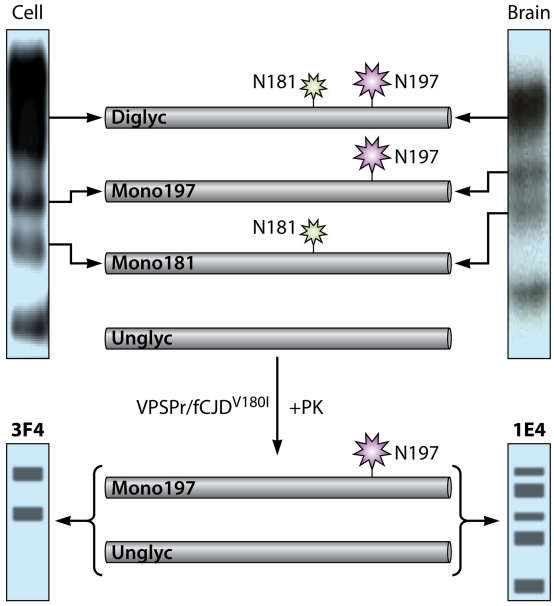

FIG 4 Schematic diagrams of the conversion of four PrP glycoforms from VPSPr or $fCJD^{V180I}$ and Western blot assays of four PrP glycoforms, including a PrP diglycosylated PrP (Diglyc), a PrP monoglycosylated at N197 (Mono197), a PrP monoglycosylated at N181 (Mono181), and an unglycosylated PrP (Unglyc) from cultured cells expressing human PrP^{V180I} or from the brains of VPSPr patients without PK treatment. In VPSPr or $fCJD^{V180I}$, only Mono197 and Unglyc are converted into a PrP^{Sc} that exhibits two bands by 3F4 and five bands by 1E4. In contrast, Diglyc and Mono181 are not converted into PrP^{Sc}.

GPI ANCHOR AND FORMATION AND INFECTIVITY OF PRIONS

GPI Anchor on PrP^{C} and PrP^{Sc}

PrP is usually tethered to the neuronal cell membrane through a GPI anchor at residue 231 of the protein (38, 170). When treated with phosphoinositide-specific phospholipase (PI-PLC), the GPI anchor of PrP^{C}, but not PrP^{Sc}, is cleaved, causing PrP to be released from the plasma membrane of cultured cells (170, 171). Murine PrP molecules carrying mutations linked to fCJD, GSS syndrome, and FFI are partially resistant to phospholipase cleavage in Chinese hamster ovary cells (172). These findings indicate that prion infection and PrP mutation may result in changes to the anchor structure.

Effect of the GPI Anchor on Biosynthesis and Trafficking of PrP

As mentioned earlier, in addition to N-linked glycans, the GPI anchor is a functionally critical domain in the structure of the PrP molecule. To determine the effect of the anchor on the function of PrP, the synthesis and trafficking of anchorless PrP^{C} ($PrP\Delta GPI$) were extensively investigated in Fischer rat thyroid cells (173). As seen in anchored PrP^{C} controls, the $PrP\Delta GPI$ protein exhibited a

similar banding pattern, with three major bands corresponding to un-, mono-, and diglycosylated isoforms. These observations, however, are discordant with earlier findings by other groups (174, 175). In the latter study by Chesebro et al., it was shown that anchorless PrP had an underrepresentation of glycosylated forms (175). Interestingly, the study by Campana et al. suggests that this discrepancy could result from either distinct conformations of different isoforms or by particular sugar modifications that prevent antibody recognition of epitopes (174). This is supported by the fact that highly glycosylated PrPΔGPI was detected with SAF32 and SAF61 antibodies but not with the PRI308 antibody, which has an epitope similar to that of the 3F4 antibody. Indeed, Campana et al. also found that the mutant protein displayed different oligosaccharide chains compared to normal PrP^{C}, suggesting that proper PrP glycosylation is contingent upon an intact GPI anchor. However, the anchorless protein that was tethered to the plasma membrane did not adopt a transmembrane conformation. Furthermore, unlike anchored PrP^{C}, which is localized to the cell membrane, most (>90%) of the anchorless PrP^{C} either accumulated in the Golgi apparatus or was released into the culture medium (173) (Fig. 2). The authors proposed that the inability of anchorless PrP to localize to the cell surface could explain why the symptoms of the disease in anchorless PrP-expressing Tg mice are minimal. They suggested that the cell surface may be the critical site for the initiation of neurodegeneration, given that anchored PrP^{C} on the cell membrane functions as a signaling molecule (173).

Effect of the GPI on Conversion of PrP^{C} into PrP^{Sc}

Several studies have evaluated the role of the GPI anchor in PrP^{Sc} formation and have yielded various results among cell-free and cell culture models. In cell-free experiments, anchorless PrP can be efficiently converted to the PrP^{Sc} form (176, 177). In one study using mouse neuroblastoma cells (ScN2a), expression of a C-terminally truncated PrP that lacked the GPI anchor did not inhibit PrP^{Sc} formation, but the PrP^{Sc} that resulted also lacked the GPI anchor (174). However, most studies using cell culture models have revealed that the GPI anchor is required for prion formation. For instance, McNally et al. reported that cells expressing anchorless PrP^{C} did not support persistent infection in scrapie-infected cells (178). Furthermore, Pl-PLC treatment reduced PrP^{Sc} formation and even cured prion infection in ScN2a cells (170, 179). By fusing PrP to a transmembrane domain, PrP^{C} redirected to clathrin-coated pits on the cell surface, which also prevented the formation of PrP^{res} (180). Treatment with glimepiride, a sulfonylureal antidiabetic agent previously showed to upregulate endogenous PI-PLC (181), significantly decreased the level of PrP^{Sc} in three infected neuronal cell populations (SMB, ScGT1, and ScN2a) (182).

In 2005, Chesebro et al. generated Tg anchorless PrP mice and infected them with three scrapie strains (RML, ME7, and 22L) (175). Although the inoculated mice were virtually asymptomatic with a prolonged life span, PrP^{res} amyloid plaques exhibiting pathological features reminiscent of AD were detectable in their brains. It is worth noting that mice heterozygous for anchorless and wild-type PrP had an acceleration of the scrapie disease. Further studies of these mice showed that PrP^{res} could be detected in the blood, spleen, and heart. Interestingly, such mice suffered from an amyloid heart disease characterized by restrictive cardiomyopathy (183). The aforementioned findings were observed in heterozygous Tg mice expressing only anchorless PrP. However, the same group later reported a novel fatal condition with distinct clinical features and intracranial pathology in homozygous Tg mice expressing 2-fold more anchorless PrP (184). Brain tissue of these Tg mice exhibited high infectivity and revealed dense amyloid PrP^{res} plaque deposits without spongiform degeneration, while infected non-Tg animals showed nonamyloid PrP deposition with prominent spongiform degeneration (184). In summary, the above-described studies indicate that the GPI anchor itself may not be required for prion formation; however, it may play an important role in keeping PrP^{C} sustainedly available for prion propagation on the cell surface, thereby contributing to prion neurotoxicity and subsequent spongiform degeneration.

It has been suggested that the endosomal recycling compartment or caveola-like membranous domain is where the conversion from PrP^{C} to PrP^{Sc} takes place (184, 185). By analyzing the ratio of PrP^{C} to PrP^{Sc} in various cell populations in which protein trafficking has been selectively inhibited, Marijanovic et al. were able to deduce that conversion is unlikely to occur in early or late endosomes but more likely to occur in the recycling compartment (186). Interestingly enough, this compartment plays a major role in the internalization of GPI-anchored proteins (187). Consistent with the findings of Chesebro et al., this study supports the hypothesis that the GPI anchor on PrP^{C} is involved in conversion to PrP^{Sc} if, indeed, conversion takes place at the recycling compartment level.

Effect of the GPI anchor on PrP^{Sc} Infectivity

Although an intact GPI anchor may be necessary for PrP^{C} to be incorporated into the growing PrP^{Sc} reaction, the GPI anchor of the infectious PrP^{Sc} molecule may not affect its virulence. In a study employing prion-infected murine brain homogenates, by using cathepsin D, Lewis and colleagues were able to eliminate a short sequence at the C terminus of PrP^{Sc} to which the GPI anchor is attached (188). As determined by N2a cell-based and mouse bioassays, this truncated PrP^{Sc} had no effects on prion titers or PrP^{Sc} amplification *in vitro*. In light of these observations, the authors suggested that the GPI anchor of PrP^{Sc} has little or no role in either the propagation or infectivity of prion agents. The finding that prion infectivity does not require the GPI anchor is consistent with observations by several groups, including Chesebro and others mentioned above (98, 184, 188, 189). Importantly, however, the *de novo* generation of prion infectivity can be achieved with rPrP alone. Infectious PrP fibrils generated from rPrP engendered prion disease not only in Tg mice overexpressing PrP but also in wild-type animals (189).

DE NOVO GENERATION OF PRIONS WITH RECOMBINANT PRION PROTEIN

It has been suggested that the "protein-only" hypothesis will be definitively proved when prion disease is induced in a natural host by an infectious prion protein generated *in vitro* (190). Several recent studies have, indeed, generated transmissible prion diseases with rPrP in natural hosts (96, 97, 100, 189, 191–194). These infectious recombinant prions were generated with or without the application of serial PMCA (sPMCA) (96, 97, 99, 100, 189, 191–198). The sPMCA-dependent generation of prions from rPrP requires additional cofactors such as RNA or lipids (193, 194). Kim et al. reported that PMCA-amplified $rPrP^{PMCA}$ from 263K-seeded hamster rPrP caused prion disease in hamsters, although the in-

cubation time and attack rates were highly variable (191). Studies by Makarava and colleagues showed that $rPrP^C$ could be converted to amyloid fibrils without sPMCA by using primarily guanidine hydrochloride and other reagents (100). In order to acquire infectivity, these amyloid fibrils were subjected to annealing with either normal brain homogenate (NBH) or bovine serum albumin (BSA). The resulting mixture was inoculated intracerebrally into wild-type Syrian hamsters. This process engendered infectious prions in a small fraction of asymptomatic animals; however, the clinical disease was observed only at the second or third serial passage (100). At these stages, the resultant diseased phenotypes exhibited distinct clinical and neuropathological features characterized by a slowly progressive clinical course (comparable to human prion diseases) and by PrP^{Sc} plaque deposition in subpial and subependymal areas of the CNS. All of the animals inoculated with NBH-annealed rPrP fibrils exhibited clinical signs of disease and contained PK-resistant PrP^{Sc}. In contrast, none of the animals inoculated with BSA-annealed rPrP fibrils showed clinical signs, although PK-resistant PrP^{Sc} was detected in six out of seven animals (100). Even though rPrP amyloid fibrils also became infectious without the addition of cofactors, it is worth noting that their infectivity seemed to be low (98, 99, 192). Therefore, most of the above-described studies indicate that cofactors are important for rPrP to become infectious prions.

DETECTION OF PRIONS

Traditional Approaches

Prion diseases are almost exclusively diagnosed on postmortem histopathological analysis aiming to identify characteristic features of prionopathies, including astrocytosis, neuronal loss, spongiosis, and deposition of misfolded PrP^{Sc}. However, for a definitive diagnosis, it is imperative to detect PrP^{Sc} by either *in situ* IHC or Western blot probing with antibodies directed against PrP. These methods have become routine tools for the diagnosis of prion diseases. To differentiate PrP^{Sc} from PrP^C by IHC, the paraffin-embedded and formalin-fixed tissue sections are often heated and pretreated with formic acid in order to eliminate PrP^C immunoreactivity and reveal PrP^{Sc} deposits. By Western blotting, PrP^{Sc} is differentiated from PrP^C after brain homogenates are pretreated with PK. Since PrP^C is PK sensitive and PrP^{Sc} is partially PK resistant, no PrP^C can be detected in the uninfected brain homogenates, leaving only PK-resistant PrP^{Sc} available for detection.

Although the first clinical diagnostic criteria for human prion disease were proposed 30 years ago, they have been updated to reflect recent advances in technology and have incorporated the use of brain imaging, as well as surrogate biomarkers in the cerebrospinal fluid (CSF) (199–203). The clinical diagnostic criteria for probable CJD include typical periodic sharp- and slow-wave complexes on the electroencephalogram, increased tau and 14-3-3 proteins in the CSF, and hyperintense signal changes in the basal ganglia, thalamus, and cortical areas on magnetic resonance imaging (203). With respect to the differential diagnosis of prion diseases, the most common diseases should be considered first, including AD and Lewy body dementia in elderly patients and chronic inflammatory CNS disorders in younger patients (203).

Protein Misfolding Cyclic Amplification (PMCA)

A major barrier to studying prions is their long incubation times. Infectivity studies are largely impractical, as it takes several months for prions to reach sufficient titers in the CNS before animals become symptomatic. The study of infectious prions has been greatly ameliorated with the introduction of *in vitro* cell-free assays. Caughey and coworkers developed the first cell-free PrP conversion assay by using PrP^{Sc} purified from scrapie-infected brains as a template and NBHs as a substrate (176). Since it requires an excess of PrP^{Sc} to convert a minute amount of radiolabeled PrP^C, this assay was used mainly to study the molecular mechanism of PrP^{Sc} propagation (204). Nevertheless, it is this assay that provides the first evidence that *in vitro* PrP conversion can be accomplished by utilizing a cell-free system. By using these principles, Soto and coworkers developed a PMCA assay (205). This method allows the rapid and efficient amplification of PrP^{Sc} seeds from PrP^C substrates, thereby generating infectious particles that retain their infectivity (206, 207). First described in 2001, PMCA has undergone several revisions, although the underlying principle has remained the same: seeding infectious particles through a template-directed mechanism involving the conversion of PrP^C to its infectious counterpart (205). PMCA is able to amplify extremely small quantities of PrP^{Sc} and may be reflective of the *in vivo* mechanism of replicating prion proteins. Thus, it is a useful tool for the study of prion propagation and for the detection of PrP^{Sc}.

PMCA is based on the principle of PrP^{Sc} polymerization, analogous to the PCR method that is used to amplify DNA. PrP^{Sc} serves as a template for recruiting PrP^C into the growing oligomer, and this is similar to the way amyloid fibrils form in other neurodegenerative conditions (208). In PMCA, small quantities of PrP^{Sc} are incubated with excess PrP^C, causing PrP^{Sc} polymers to form. These polymers are then subjected to several cycles of sonication, which shears them apart; this effectively produces more available polymers capable of catalyzing the conversion of further PrP^C (207). This process takes approximately hours to days to complete and exponentially amplifies prion proteins after each cycle. PrP^{res} particles generated by *in vitro* PMCA have been shown to have several physiochemical and structural properties similar to those of PrP^{res} derived from infected brains (206).

Three major modifications of PMCA have been made to further increase the efficiency of this method. The first modification was automation, which increases the efficiency and sensitivity of conversion (209). The second modification was the addition of Teflon beads, which enhances the conversion to PrP^{Sc} and increases the sensitivity of prion detection (210). The third modification of the PMCA protocol supports the amplification of hamster PrP^{Sc} in the presence of recombinant hamster PrP instead of using brain PrP^C as a substrate (211).

Since its application, PMCA has grown to be applicable to numerous methodologies in the study of prions. As PrP^{Sc} remains the most widely acceptable marker for the diagnosis of TSEs (212), PMCA has served as a useful diagnostic tool for prion diseases. Recently, a quantitative PMCA assay has been devised to assess the concentration of PrP^{Sc} in the CNS, spleen, and bodily fluids such as blood and urine (213). Its potential as a clinically valuable tool comes from its high sensitivity, which is important for the small amounts of PrP^{Sc} present in nonneural tissues during presymptomatic phases of the disease (212). Through PMCA, PrP^{Sc} has been detected in blood during the early stages of scrapie infection of hamsters, demonstrating that PMCA is a promising tool that may eventually serve as a rapid clinical test for TSE diagnosis in humans (214, 215).

PMCA may be readily used to investigate prion strains and probe the intricacies of the prion species barrier. By combining PrP^{Sc} and PrP^{C} from different species and evaluating whether infectious particles are generated, it has been suggested that PMCA may be a reasonable complement to *in vivo* species barrier studies and strain generation studies (216–220). Furthermore, PMCA has been used by several groups to determine the involvement of other cofactors, such as divalent metal cations and polyanions, in the conversion of cellular to infectious prion proteins (96, 195). Lastly, PMCA has been applied as a method to rapidly assay molecules that may inhibit the conversion of PrP^{C} to PrP^{Sc} (221, 222).

Amyloid Seeding Assay (ASA)

Another such method that has been used to detect prions is the ASA (223). First described in 2007 for the brains of CJD patients, the ASA utilized partially purified prions to seed bacterially generated rPrP into polymerizing amyloid fibers. Such formation was detected by a fluorescence shift (from 342/430 to 442/482 nm) with thioflavin T (ThT), a marker of amyloid formation. Interestingly, the sensitivity of this assay was dependent upon the strain type, and in certain strains, the assay was capable of detecting prions amounts as small as 1 fg.

The ASA is capable of ultradetecting prions in a rapid manner—on the order of 1 day (potentially decreasing costs). PMCA is sonication dependent; it has a high percentage of false positives and negatives and is better at detecting certain strains than others (223). Therefore, ASA may be superior to PMCA in detecting prion strains. Moreover, ASA has been able to detect both $sPrP^{Sc}$ and $rPrP^{Sc}$ species in hamsters and mice (223). This is useful in overcoming detection barriers with traditional Western blot assays and enzyme-linked immunosorbent assays, which rely on PK resistance, thereby precluding the detection of $sPrP^{Sc}$. The advantage of ASA was further delineated in a study showing that infectious $sPrP^{Sc}$ was produced *in vitro* during the polymerization of rPrP (196).

ASA has been applied to other diseases, including HD, a neurodegenerative disease that is characterized by trinucleotide CAG repeats in the huntingtin protein (HTT) and results in a variety of motor and cognitive symptoms (224). Similar to the ASA for prion disease, partially purified HTT substrates were capable of recruiting misfolded HTT into a growing amyloid fibril (225). Additionally, amyloid seeding assays have been used to detect Aβ aggregates by using a kinetic nucleation reaction *in vitro* (226).

RT-QuIC

Recently, a novel assay used in the antemortem diagnosis of sCJD was developed by Atarashi et al. and Caughey et al. (227, 228). Known as real-time quaking-induced conversion (RT-QuIC), this methodology is capable of detecting as little as 1 fg of PrP^{Sc}. The RT-QuIC assay combines a small volume of a test sample (approximately 2 to 15 μl) with a reaction mixture containing rPrP substrate, the amyloid-sensitive dye ThT, and a detergent or chaotropic agent. ThT fluorescence is then measured in real time and detects any fluorescence increase caused by fibrillization of the rPrP substrate. Throughout this process, the 96-well plate is incubated in a temperature-controlled fluorescence microplate reader and is subjected to several cycles of vigorous quaking (229).

Using the cerebrospinal fluid of sCJD patients, this assay achieved 95.8% sensitivity and 100% specificity in the detection of prions (227–231). RT-QuIC has also been used to estimate the concentration of prions in a rapid fashion—on the order of 2 days—in sheep (scrapie), deer (CWD), and hamsters (in TME and scrapie) with sensitivities that parallel those of other prion assays (232). Remarkably, a recent improvement to the assay has allowed for the swift detection of prion seeds in 4 to 14 h (231). Using nasal brushings, RT-QuIC has been used in the clinical setting to diagnose CJD with 97% sensitivity and 100% specificity (232). More recently, Orrú et al. further advanced the RT-QuIC assay by using recombinant bank vole PrP as a universal substrate and demonstrated its efficacy in detecting various prion strains (233). Not only did they successfully detect prion seeding activities from 28 human and animal prion strains at a 10^{-4} seed brain homogenate dilution, but they also discriminated classic and atypical BSE, scrapie, sCJD, and vCJD by using bank vole PrP or hamster-related PrP. Because of its sensitivity, convenience, and suitability for large sample sizes, RT-QuIC has been adopted by numerous surveillance centers around the world as a diagnostic test for sCJD (227–230, 234, 235). Indeed, this promising assay may revolutionize the preclinical diagnosis of prion diseases.

THERAPEUTICS OF PRION DISEASE

Sulfated Polyanions and Heparin Mimetics

There are currently no available therapeutics capable of reversing or even limiting the progression of human prion diseases. Early studies demonstrate a prolongation of prion incubation times by using various sulfated polyanions (HSPGs) in scrapie-infected hamsters and mice and in cultured neuroblastoma cells (236–240). Through a similar mechanism, Congo red, a metachromatic dye, was found to inhibit PrP^{res} accumulation and reduce scrapie infectivity in neuroblastoma cells (241, 242). More recently, similar agents known as heparin mimetics have shown promising results *in vitro* in that they inhibit prion propagation in scrapie-infected cells (243). Presumably, these agents compete with endogenous cellular HSPG (a potential enzymatic cofactor) and bind to PrP^{C}, thereby decreasing its subsequent conversion to PrP^{Sc}. However, newer heparin mimetics with minimal toxicity, such as CR36, have not recapitulated these findings *in vivo* (244). Other classes of drugs that have exhibited antiprion activity include tetrapyrroles (245, 246), branched polyamines (247, 248), lichens (127), and β-sheet breaker peptides (249).

Antimicrobial Therapy

Several antimicrobial agents have been extensively studied as therapies for prion disease. Quinacrine, a tricyclic antiprotozoal therapeutic, has been observed to decrease PrP^{res} accumulation in ScN2a cells (250); however, its efficacy *in vivo* and in human trials has been limited (221, 251, 252). Although some studies employing small cohorts of patients have showed modest improvements in symptoms (253, 254), these clinical trials have failed to show any decrease in the mortality rate (reviewed in reference 255). In addition to quinacrine, another tricyclic compound with an aliphatic side chain is chlorpromazine, which has also been evaluated as a potential therapy for prion disease. Both chlorpromazine and quinacrine have shown to decrease PrP^{Sc} formation in prion-infected cell cultures (256). However, none of the case studies utilizing chlorpromazine have shown an improvement in symptoms or a benefit in survival (257, 258).

Other antimicrobials that have been studied in prion diseases are tetracycline antibiotics. *In vitro*, tetracyclines have demon-

strated a variety of antiprion activities, ranging from inhibition of PrP amyloid fiber assembly, decreasing the protease resistance of PrP^{Sc}, and prevention of PrP-induced neuronal death and astrocyte proliferation (259). *In vivo*, scrapie-infected Syrian hamsters treated with various tetracycline compounds exhibited a delayed onset of symptoms and prolonged survival times (260, 261). Recently, a double-blind, randomized, controlled trial of the use of doxycycline in CJD patients was conducted in Italy; however, this study did not reveal any mortality rate benefit in patients receiving doxycycline rather than a placebo (262). Anthracycline, a tetracycline chemotherapy agent that also binds to amyloid fibrils and prevents amyloid fibrillization *in vitro*, has also been shown to delay symptom onset and lengthen survival in scrapie-infected Syrian hamsters (263).

Given that prion diseases were originally perceived as "slow virus" infections, it is no surprise that in early studies, acyclovir (264, 265), vidarabine (266), interferon (267), and amantadine (268) were evaluated as potential prion therapies. Perhaps the best studied of these antivirals was amantadine, which demonstrated variegated responses in human trials (reviewed in reference 255). Another well-studied antimicrobial agent used in the treatment of prion diseases is amphotericin B (AmB). Some data suggest that the antifungal agent AmB and its analogue MS-8209 are capable of prolonging prion incubation times, decreasing PrP^{Sc} accumulation in the CNS, and delaying the appearance of pathological features of the disease (269–272). *In vitro*, this polyene antibiotic has been shown to block the formation of abnormal PrP isoforms, presumably through its effect on PrP trafficking in detergent-resistant microdomains (273). Moreover, treatment with AmB or MS-8209 has been shown to prolong survival in scrapie-infected mice and in Syrian hamsters infected with certain scrapie strains (274–276). Although no large-scale human trials were conducted with AmB, one study failed to show a survival benefit in CJD patients treated with AmB (277).

Small Molecules and Other Agents

Apart from antimicrobials, other agents have been found to prolong survival in TSE-infected animals. One of these, astemizole, a second-generation antihistamine drug, was shown to inhibit prion replication and contribute to prion clearance by upregulating autophagy in neuroblastoma cells (278). Moreover, several small-molecule compounds have had variable success when tested in animal models. Among them, curcumin, the main ingredient of turmeric, and memantine, a drug used in the treatment of moderate-to-severe AD, have shown modest increases in survival when administered to scrapie-infected mice (279). *In vitro*, curcumin was observed to decrease PrP^{res} in scrapie-infected neuroblastoma cells and inhibit the conversion of PrP^{C} to PrP^{Sc} (280).

With regard to human prion diseases, several therapies have been tested that have met with little clinical success. Flupirtine, a nonopioid analgesic, has been evaluated as a treatment for CJD, as it showed some neuroprotective effects *in vitro* (281, 282). However, in a double-blind study, while flupirtine demonstrated some improvement in cognitive function, it did not show any increased survival benefit (283).

Immunotherapy and New Approaches

Recent therapeutic approaches have focused on immunotherapy with monoclonal antibodies. When bound to PrP^{C}, these antibodies prevent the pathological transformation to PrP^{Sc}. When the anti-PrP monoclonal antibody 6H4 was introduced into scrapie-infected cultures, there was a significant reduction in PrP^{Sc} (179). Furthermore, other targeted antibodies have been shown to increase PrP^{Sc} turnover, abrogate prion replication, and clear existing PrP^{Sc} molecules (284, 285). More recently, anti-PrP antibodies capable of traversing the blood-brain-barrier were developed, allowing for the noninvasive delivery of these molecules (286). These findings have been replicated *in vivo*, in which administration of monoclonal antibodies decreased peripheral levels of PrP^{Sc}, as well as prolonged the survival of scrapie-infected animals (287). Moreover, Tg mice expressing anti-PrP monoclonal antibody 6H4 were protected against scrapie infection in the presence of these antibodies (288). Such immunotherapeutic strategies have stimulated an interest in generating vaccines that confer passive immunization with antibodies against PrP (289).

Additional strategies have probed into stabilizing the PrP^{C} substrate, thereby preventing its conversion to PrP^{Sc}. Molecules such as 2-pyrrolidin-1-yl-*N*-[4-[4-(2-pyrrolidin-1-yl-acetylamino)-benzyl]-phenyl]-acetamide, also known as GN8, bind to unstable residues in PrP^{C} and have been shown to decrease the amount of converted PrP^{Sc} *in vitro* and prolong survival in TSE-infected mice (290). Other molecules, such as anle138b, have been shown to inhibit the creation of pathogenic PrP oligomers *in vivo* and *in vitro* (291). In recent years, novel therapeutic strategies have employed RNA interference to knock down PrP^{C} levels, thereby depleting the substrate for PrP^{Sc} (292, 293).

Inhibitors of the Unfolded-Protein Response

Moreno and coworkers recently reported that oral administration of GSK2606414, a targeted inhibitor of the protein kinase RNA-like ER kinase (PERK), abolished the development of clinical prion disease in mice (294). This therapeutic effect was observed in mice treated at both early and late stages of the disease. The PERK inhibitor is a significant mediator of the unfolded-protein response (UPR) pathway, as it prevents UPR-mediated translational repression. In neurodegeneration, prion-induced translational repression has been observed to be the key molecular event associated with a deficiency of essential proteins leading to disruption of cellular homeostasis and ultimately cell death (295). The study of Moreno et al. suggests that pharmacological inhibition of the UPR pathway may have a therapeutic effect. In fact, the UPR inhibitor is presumably effective despite the continuous accumulation of misfolded PrP (294). Given these findings, it is not surprising that targeting of the PERK pathway is believed to be a novel therapeutic strategy not only for prion diseases but also for other neurodegenerative conditions (296).

A major limitation in the development of prion therapies has been the lack of studies employing agents that are relatively devoid of toxicity and are capable of being efficacious at symptomatic phases of the disease. Early diagnosis of prion diseases would greatly assist in the swift administration of therapeutics; however, given the rapid course of the diseases, this is certainly an arduous task. Furthermore, there is some speculation that prion diseases may exhibit drug resistance properties similar to those of bacteria, viruses, and fungi (297). However, recent advances in molecular biology and neuroimaging may improve the accuracy of disease diagnosis, allowing earlier intervention (298).

BIOSAFETY OF PRIONS

Given the nature of their transmissibility and inherent resistance to degradation, great care should be undertaken when handling prions in the laboratory or clinical setting. By virtue of their physiochemical properties, prions are impervious to customary methods of inactivation and require a great deal of attention to their disposal. Prions are not inactivated by laboratory agents such as alcohol, formalin, or ammonia. Furthermore, these proteins are known to persist under extreme conditions. Methods including exposure to ultraviolent light, autoclaving under standard conditions, or boiling at high temperatures are ineffective (299). Numerous reagents and conditions have been tested (reviewed in reference 300); among them, autoclaving at 121°C for 1 h in combination with exposure to 1 N NaOH or Environ LpH appears to be particularly effective (301).

Great care should be exercised when handling such specimens in a pathology laboratory. Most prions should be handled in a biosafety level 2 (BSL2) setting. Certain prions, such as BSE prions, may necessitate the use of a BSL3 facility or at least the use of BSL3 practices in a BSL2 facility (302). Areas involving the study of infected prion tissue should have restricted access so as to minimize exposure to as few individuals as possible. General protective laboratory measures should be maintained at all times. Precautions with coveralls, face masks, gowns, gloves (preferably cut resistant), and shoe covers are standard.

It should be noted that the safety of handling these tissues varies by organ system and is dependent on the frequency at which certain organs are infected. This frequency effectively determines the infectivity of such organs and directs the level of caution needed when working with the specimen (303). Not surprisingly, the eye, pituitary gland, brain, and spinal cord pose the greatest infection risk. Lymphoid tissues, cerebrospinal fluid, and placental tissue pose a low risk. Blood, bone marrow, peripheral nerve tissue, sputum, urine, feces, tears, semen, sweat, milk, and vaginal secretions are deemed to be noninfectious or minimally infectious (303).

In addition to organ tissue, it should be noted that the exposure of intact skin to one of the above-mentioned tissues poses less risk than the exposure of open skin or the eye or inoculation into the bloodstream (304). Extra caution with double gloves or cut-resistant gloves should be used to avoid needlestick injuries to the skin. Accidental skin exposure requires routine washing with detergent and water, followed by exposure to 0.1 N NaOH for several minutes (305). Accidental exposure of the eye or other mucous membranes should prompt copious irrigation according to standard institutional procedures (306). When working with BSE prions, BSL3 practices should be employed. This includes the use of full-body protective wear and disposable equipment that can be incinerated after a single use. Furthermore, HEPA filters should be incinerated and other surfaces should be decontaminated with 1 N NaOH (307). With regard to the handling of body fluids, the World Health Organization recommends that no additional precautions are needed, as they are considered noninfectious. CSF should be handled with special care, however; any material that it comes in contact with it should be incinerated (306).

Standard methods of tissue processing, including formalin fixation and paraffin embedding, do not render prions inactive. Therefore, it is recommended that such specimens be postfixed in 96% formic acid, phenol, or 10% formalin before additional processing (307). Embedding should be done in disposable molds, and all specimens should be appropriately labeled. After slides have coverslips on them, they should be soaked in 2 N NaOH for 1 h (307). All instruments that come in contact with these tissues should be incinerated or decontaminated with NaOH.

There have been numerous cases of contamination of surgical equipment leading to iatrogenic CJD (308). Special care and precautions should be exercised in the operating room to minimize this risk. In addition to standard protective equipment, manual saws instead of power tools should be used to decrease the risk of splattering (305). Single-use instruments should be used, if possible, as reusing instruments increases the risk of iatrogenic CJD (309). This becomes especially prudent when dealing with high-infectivity organs such as the brain or spinal cord. If resources are limited, instruments should be sterilized as soon as possible. All liquids should be retained after the procedure and decontaminated, while sharps and other trash, including biological waste, should be incinerated at a temperature of 1,000°C (310). All surfaces should be disinfected with 2 N NaOH for 1 h and then cleaned thoroughly thereafter according to institutional standards. Surgical instruments should be immersed in 1 N NaOH for 1 h and then steam sterilized at high temperatures (121°C for gravity displacement or 134°C for a porous load) (307). Any additional items that cannot be steam sterilized should be soaked in NaOH.

In the clinical setting, standard precautions should be taken in the care of patients afflicted with prion disease. Human-to-human spread of such diseases via either droplet exposure or contact has not been established (311, 312). There are no additional precautions that need to be used when handling body fluids of patients with CJD. Such fluids pose zero risk of transmission of the disease (313). However, there has been much research into the blood-borne transmissibility of prion diseases. One early study, known as the Transfusion Medicine Epidemiology Review, recognized four individuals who developed vCJD after accepting blood from donors who contracted the disease (314, 315). Seven individuals developed vCJD after receiving blood from individuals who never went on to develop the disease, suggesting that one may be "subclinically infected," i.e., capable of transmitting the disease but not developing it (314). However, a follow-up study demonstrated that this is unlikely to serve as a major mechanism of vCJD development (316). With regard to the transmissibility of sCJD, one study showed that the frequency of blood transfusions 10 years prior to the onset of symptoms is much more frequent in sCJD than in other neurological diseases (317). Conversely, one study conducted in the United States showed virtually no transmissibility of non-vCJD prion diseases by transfusion (318). It is worth noting that some epidemiological studies have suggested a potential association with sCJD and a history of various non-brain-related surgeries (319–322), while others have found no correlation between surgeries and sCJD incidence (323, 324). Potentially relating to this concern, PK-resistant PrP has been identified (by Western blotting) in skin from a single case of vCJD (325). Given that PrP^{Sc} or prion infectivity has been reported in the skin of animals (326, 327), the possible skin infectivity of infected individuals should not be completely ignored.

PRION-LIKE PROTEIN AGGREGATES

Common Features of Protein Misfolding Disorders

AD, CJD, PD, HD, and amyotrophic lateral sclerosis (ALS) are all classified as human neurodegenerative disorders or prion diseases that manifest themselves in numerous ways, ranging from neurocognitive deficits to movement disorders (Table 1). Interestingly, these diseases are all characterized by the accretion and potential aggregation of aberrant proteins. Because of this feature, it has been suggested that a common molecular mechanism could unify the etiologies of these seemingly disparate diseases (328). Furthermore, this suggestion implies that the treatments for such diseases could be similar, if not identical, potentially targeting common cellular pathways that underlie all neurodegenerative diseases.

On the basis of the assumption that these diseases result from an accumulation of toxic proteins, it is no surprise that disruption of the ubiquitin-proteasome system and molecular chaperones is a key molecular feature of neurodegenerative diseases (329, 330). Commonly referred to as aggresomes, these protein aggregates form as a cellular response when proteasomes are unable to handle a high number of misfolded proteins (331). Proteasomes are large, multisubunit protein complexes found in the nucleus and cytoplasm that serve to degrade misfolded or undesired proteins. If these proteasomes fail to perform their specified function, misfolded proteins can accrue intracellularly. In each of the neurodegenerative diseases, there are prototypic molecules that aggregate; these aggregates likely play a pathogenic role. However, the exact nature and mechanism of toxicity of these aggregates are largely unknown (332).

Amyloid β (Aβ) and Tau

Interestingly, other toxic proteins have been found to behave like prions in that they can self-perpetuate in an amplification-like reaction. Particularly with regard to Aβ, prion-like Aβ strains with unique biological properties have been reported (103). Studies by Eisele et al. have shown that intracerebral introduction of β-amyloid brain homogenates was capable of precipitating cerebral β-amyloidosis in susceptible hosts (333). Furthermore, in a prion-like fashion, cerebral Aβ angiopathy developed in Aβ-precursor protein Tg mice by direct inoculation of Aβ substrates (334). These findings were revisited by Prusiner and colleagues, who used an *in vivo* luciferase assay to detect Aβ deposition (335).

Such activity is not limited to Aβ, as tau, the other toxic intracellular protein in AD, has been shown to exhibit prion-like activity as well. *In vitro*, extracellular tau is taken up by cells and subsequently undergoes a fibrillization reaction. Once taken up, these tau fibrils are capable of being propagated among cells (336). These findings are translated *in vivo*, as Clavaguera et al. demonstrated that inoculation of P301S mutant tau-expressing brain extracts into wild-type tau-expressing mice induced the polymerization of tau into filaments that migrated to nearby brain regions (337). Moreover, when Tg mice overexpressing mutant human tau (P301S) were injected with preformed full-length or recombinant tau fibrils, neurofibrillary tangle-like inclusions formed; these inclusions propagated throughout the brain in a distinct, time-dependent fashion (338). Such a defined pathological spread was also observed when using Tg mice that differentially expressed tau in the entorhinal cortex. In one study, Liu et al. were able to demonstrate that tau is capable of spreading transsynaptically (339).

α-Synuclein

PD is a late-onset neurodegenerative disease characterized by the degeneration of nigral dopaminergic neurons. This is potentially caused by an accumulation of α-synuclein in structures known as Lewy bodies and Lewy neurites. When bound to membranes, α-synuclein is found with a high α-helical structure; however, at high concentrations, mutant α-synuclein can assume a β-sheet conformation capable of forming fibrils (340). Interestingly, when embryonic dopamine neurons are transplanted into patients with PD, such neurons recapitulate pathological features of PD years later (341). On the basis of such findings, it has been suggested that host-derived misfolded α-synuclein recruits nascently produced α-synuclein to misfold (342). This is further supported by data demonstrating that α-synuclein can be transmitted via endocytosis to adjacent neurons, forming Lewy-like inclusions in both *in vivo* and *in vitro* models (343). Mougenot and coworkers demonstrated that when homogenates from PD Tg mice are inoculated into healthy mice, the inoculated mice develop motor deficits and accumulate phosphorylated α-synuclein in an infectious "prion-like" manner (344). Similar to tau propagation, inoculation of synthetic α-synuclein fibrils into wild-type non-Tg and α-synuclein Tg animals causes a cell-to-cell spread of α-synuclein in a distinct anatomic pattern (345, 346). Interestingly, intracerebral inoculation of human-derived, Sarkosyl-insoluble α-synuclein into mice induces Lewy body pathology, suggesting that α-synuclein has some prion-like properties (347).

Huntingtin

HD is a polyglutamine trinucleotide expansion repeat disorder characterized by autosomal dominant mutations in the huntingtin-encoding gene leading to progressive chorea. *In vitro*, fibrillar polyglutamine molecules form aggresomes (associated with molecular chaperones and proteasomal subunits) that recruit additional proteins in a prion-like manner (348). Conceptualizing huntingtin proteins as prions potentially offers an explanation as to why patients with expanded repeats do not manifest the disease until much later, despite the production of mutant protein during embryogenesis (349).

Cytoplasmic Polyadenylation Element Binding Protein

Several nonpathogenic mammalian prions that play several critical roles in cellular psychology have been discovered. Among these are the mitochondrial antiviral-signaling protein, the cytoplasmic polyadenylation element binding (CPEB) protein, and T-cell-restricted intracellular antigen 1. CPEB is an RNA binding protein involved in mRNA translation—specifically, in poly(A) tail elongation (350).

In addition to nonpathogenic prions, nonmammalian prions have also been identified in other organisms, namely, the yeast *Saccharomyces cerevisiae* (351). Yeast prions contain a region responsible for their prion-like activity. These nonmammalian prions generally have four characteristics in common: (i) they are rich in glutamine and asparagine residues, (ii) they exhibit conformational flexibility, (iii) they are found in both soluble and aggregated forms, and (iv) they are not necessary for proper function of their protein domains (352). Interestingly, the N terminus of the neuronal isoform in *Aplysia californica* is structurally similar to yeast prions in that it is glutamine and asparagine rich (~10%) and exhibits conformational flexibility (352). When the N-terminal domain of CPEB is fused to a glucocorticoid receptor

(GR526), it produces distinct heritable states in a prion-like manner. CPEB also forms small, prion-like aggregates capable of binding to RNA; these aggregates make up CPEB multimers that are amyloidogenic. Furthermore, similar to prions, CPEB can exist in two dominant states: biologically active multimers and inert monomers (352).

When the neuron is synaptically activated with serotonin, CPEB increases and forms punctate aggregates. It has been suggested that posttranslational modifications could facilitate this process (353). When CPEB is overexpressed in sensory neurons, several multimers are formed at the neurite and synaptic area. However, unlike prions, in which conversion from PrP^{C} to PrP^{Sc} is spontaneous, repeated pulses of serotonin induce the conversion of CPEB to its multimeric state. Such a role has implications for long-term memory (353).

CONCLUSION AND PERSPECTIVES

The road to our modern understanding of prion diseases has been full of fascinating twists and turns. It took hundreds or even thousands of years to propose that the causative agent in TSEs was a "slow" or "unconventional" virus (354, 355). Less than 30 years since the virus hypothesis, Stanley B. Prusiner and coworkers successfully identified the first infectious protein in 1982 (1). This landmark finding validated the "protein-only" hypothesis first outlined by the mathematician John Stanley Griffith in 1967 (356, 357). Since Prusiner first coined the term "prion" several decades ago, the general understanding of prions has markedly expanded. From his initial definition of "proteinaceous infectious particles," prions are now viewed as "proteins that acquire alternative conformations that become self-propagating" (1, 349) (Table 1). As a result, prions are not only associated with TSEs but are also crucial players in other neurodegenerative disorders, including AD, PD, HD, and ALS (Table 1). Furthermore, they may even play beneficial roles in the human body under physiological conditions (reviewed in reference 358).

Given recent developments, not only should the definition of prions be revised, but the concept of prion diseases should also be revisited. As designated by its name, two essential characteristics should be present in all cases of TSE: (i) transmissibility and (ii) spongiosis. However, confusion arises when facing a condition that lacks one or both features of TSEs. For instance, in a large-scale nonhuman primate bioassay, 10% of sCJD and 32% of familial prionopathies were not transmissible (359). This result was echoed by a new transmission study employing Tg mice expressing human prion protein in which sCJDMM2 and sCJDVV1 exhibited poor transmissibility compared to that of other subtypes of sCJD (118). In addition, most GSS syndrome cases (with the exception of those characterized by the PrP^{P102L} mutation) are difficult to transmit to rodents; therefore, it is suggested that CJD and GSS syndrome be divided into two groups: (i) readily transmissible and (ii) difficult to transmit or nontransmissible (360). Furthermore, the spongiosis typical of TSEs is not always detectable in GSS syndrome cases with the P102L mutation, although PrP^{Sc} and PrP-amyloid plaques are frequently observed in the CNS (361). Moreover, inoculation of Tg mice expressing murine PrP^{P101L} (comparable to human P102L) with brain homogenates from GSS syndrome cases devoid of spongiosis did not produce clinical symptoms or spongiosis; however, PrP amyloid formation was noted (152).

In addition to GSS syndrome, another disease that may not meet the TSE criteria is the newly discovered disease VPSPr, which is associated with a unique PrP^{Sc} strain that has immunoreactivity similar to that of $iPrP^{C}$ by virtue of the 1E4 antibody (27, 63, 64, 73, 74). The transmissibility of VPSPr has recently been found to be limited on first passage and virtually absent on second passage (362, 363). The aforementioned diseases that lack transmissibility, spongiosis, or both should not be considered TSEs; they should, however, be considered prion diseases because of the presence of misfolded PrP. However, the possibility cannot be excluded that VPSPr, $fCJD^{V180I}$, and the GSS syndrome represent a prion protein disorder similar to AD, given their lack of overt transmissibility and clinically symptomatic cases.

The above-described situations should lead one to question whether prion diseases should be reclassified, especially in light of the revised definition of prions (364). Under a new classification scheme (349), prion diseases should encompass all of the conditions that are characterized by the accrual of abnormal proteins—not just those limited to various PrP^{Sc} isoforms. Importantly, these proteins should be able to self-propagate and spread. This group should also include other CNS proteins, such as tau or huntingtin, and should be irrespective of classic transmissibility or the capacity to cause spongiform degeneration. Given our current understanding of these disorders, the spectrum of prion disease should be broader and more inclusive than the narrow range of conditions that fell under the tradition definition of TSE.

In the coming years, areas that are crucial for further study include the molecular mechanisms of prion formation and spread, the physiological role of PrP^{C}, prion neurotoxicity and infectivity, and prion disease therapeutics. Since the prion-like spread of misfolded proteins may be a common mechanism for all neurodegenerative disorders, prion diseases seem to be the prototype of those diseases. Therefore, it is conceivable that a complete understanding of prion diseases may be the key to understanding neurodegenerative disease as a whole.

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